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"A Better Way to Excise Inhibitory Molecules (CSPGs) from a Spinal Cord Injury Scar to Promote Regeneration"

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14. ABSTRACT
A major consequence of spinal cord injury (SCI) is the development of a glial scar. Although the scar has benefits for tissue repair, it also blocks neural regeneration. Inhibitory **chondroitin sulfate proteoglycans (CSPGs)** are elevated in the glial scar and are a major deterrent to successful regeneration. In the present study, we have demonstrated that injured astrocytes produce a wide variety of inhibitory CSPGs. To develop a more efficient method to accomplish CSPG degradation (than the bacterial enzyme chondroitinase), we are addressing a normally occurring catabolic protein for CSPG degradation, the neural aggrecanase, ADAMTS-4. We employed a two-prong approach, using studies both *in vitro*, and *in vivo*. We produced recombinant ADAMTS-4 protein and used this enzyme to reduce CSPG inhibition in experiments both *in-vitro*, and *in vivo*. We have developed critical reagents (lentivirus) and methods, and have tested these in proof of principle assays, as well as in a dorsal SCI in a rodent model. We are conducting behavioral assessments to validate the success of aggrecanase treatment, and have data indicating that aggrecanase successful ameliorates some of the negative consequences of dorsal SCI injury. The final stage of this study (no cost extension) will be to repeat the aggrecanase tests, combining it with chondroitinase ABC. The significance of these studies is the development of an efficient means by which to attenuate axonal inhibition, and thereby promote plasticity and regeneration of adult neurons following SCI.

15. SUBJECT TERMS
astrocytic scar; CSPGs; recombinant ADAMTS-4; dorsal hemisection model; pellet retrieval task

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Introduction

Subject. Spinal cord injury (SCI) is a devastating condition affecting as many as 306,000 individuals in the US alone *(http://www.brainandspinalcord.org/spinal-cord-injury/statistics.html; as of April 7, 2014)*. Beyond US soil, SCI is an all-too-common result of military combat – all with an enormous emotional, social, and financial cost to individuals and to society. Despite much needed attention over the past few decades and some significant advances, the cellular and molecular mechanisms leading to SCI are not yet clear. *Purpose:* A major consequence of SCI is the development of an astrocytic glial scar. Although the scar has benefits for tissue repair, it also blocks neural regeneration. Inhibitory **chondroitin sulfate proteoglycans (CSPGs)** are elevated in the glial scar and are a major deterrent to successful regeneration. To develop a more efficient method to accomplish CSPG degradation (than the current use of the bacterial enzyme chondroitinase), we have studied a normally occurring catabolic protein for CSPG degradation, the neural aggrecanase, ADAMTS-4. *Scope:* We used a two prong approach – employing studies both *in vitro*, and *in vivo*. We isolated, purified and tested aggrecanase on astrocytes in tissue culture; and tested aggrecanase constructs in an injury model *in vivo*, to ameliorate CSPG-induced inhibition. Studies conducted to date show a strong correlation between treatments with ADAMTS-4 and reduced sensory and motor behavioral deficits following a dorsal SCI in a rodent model. Although combination studies with chondroitinase have not yet been accomplished, the groundwork is set for this final set of experiments, and present foundation funding will allow continuation to complete this work.

The following is a Final Report for CDMRP grant SC090248, entitled "A Better Way to Excise Inhibitory Molecules (CSPGs) from a Spinal Cord Injury Scar to Promote Regeneration". Each point of progress is addressed in relationship to the Statement of Work provided in the original application.

Statement of Work

Overarching goal: To successfully degrade aggrecan and related CSPGs using naturally occurring ADAMTS---4, alone and in combination with the current "gold standard" (chondroitinase) to attenuate axonal inhibition, and promote plasticity and regeneration of adult neurons of the CNS.

Task 1. We will determine if aggrecanase and chondroitinase-mediated degradation of CSPGs produced by primary rat cortical astrocytes will foster neurite outgrowth in vitro.

1a. We will confirm that primary astrocytes upregulate CSPGs in vitro in response to injury (experimentally induced by trauma and/or administration of TGF-ß (Smith and Strunz, 2006). We will also catalogue the specific PGs upregulated and their time course (months 1-4).

Neonatal rat cortical astrocyte cultures were prepared and expanded to 16 T-75 flasks grown to near confluency. The cultures were incubated for 3 days with 2.5 ng/ml TGF-ß in 5 ml of medium. Medium was collected from 16 flasks, and guanidine HCl was added to a final concentration of 4M. The cell layers were washed with PBS, and were lysed in 0.05M sodium acetate, pH 5.8, containing 4M guanidine HCl, 0.5% CHAPS and protease inhibitors. An additional two flasks of cells were lysed, and used for the preparation of total cellular RNA, for quantitative real time PCR analysis of proteoglycan mRNA. Both medium and cell layer samples were chromatographed on Sephadex G-50 equilibrated with 0.05M sodium acetate, pH 6.0 containing 0.5% CHAPS, 0.15 M sodium chloride, and 8M urea. The void fractions were applied to a column of DEAE Sephacel equilibrated in the same buffer. The column was washed, and proteoglycans were eluted using a 0.15 to 1.0 M sodium chloride gradient (**Figure 1A**). Chromatography of medium and cell layers from astrocyte cultures each revealed several peaks that were pooled, buffer-exchanged to remove urea, and concentrated. The multiple peaks are likley due to different degrees of sulfation of these proteoglycans. Dot blot analyses of pooled fractions using antibodies specific for different CSPGs were performed (**Figure 1B**). Dot blots were reacted with antibodies to aggrecan, neurocan, phosphacan, NG2, brevican, versican, G3 domain of aggrecan, and aggrecan CS-2 domain aggrecanase-generated neoepitopes GELE and KEEE (mixture of both

antibodies). Analysis of the dot blot assays indicates that the isolated pools are largely mixtures of genetically distinct proteoglycans. Relevant to this study, we detected aggrecanase generated fragments in the major peak of medium-derived PGs eluting from the DEAE column (reacting with GELE/KEEE antibodies) which was also reactive with an antibody to the aggrecan G3 domain (also consistent with CS-substituted fragments having an intact C-terminus. This pool appeared to be highly inhibitory to neurite outgrowth (see Figure 4C). All of these proteoglycan pools are being further characterized for sulfation patterns using HPLC sulfated disaccharide analyses (analysis in progress at Glycotechnology Core Resource, UC Davis). These PG fractions have been used for analysis of neurite outgrowth inhibitory activity using an assay developed in our laboratory.

We optimized a commercially available assay for high-throughput neurite outgrowth analysis (Cellomics Neurite Outgrowth Kit) to measure the inhibitory effects of substratum bound proteoglycans on neurite outgrowth. In this assay, NeuroscreenTM-1 (NS-1) cells, a derivative of the PC12 cell line, were plated in a 96-well plate, and each well was treated with increasing concentrations of NGF to stimulate neurite outgrowth (**Figure 2**). To test the effect of CSPGs on outgrowth, plates were incubated with serial dilutions of proteoglycan prior to plating. Adsorbed PG was quantified by ELISA assay on duplicate plates. Following two days of outgrowth, cells were fixed and stained to reveal nuclei and neurites, which were detectible at different fluorescent wavelengths. A microscope with a motorized, computer-driven stage was used to obtain multiple images per well of nuclei and neurites. These images were analyzed using the Neurite Tracer plugin with Image J software. Neurite outgrowth normalized to cell number was plotted vs. NGF concentration for each concentration of proteoglycan adsorbed to the plate surface. In this manner, we obtained curves to quantify neurite inhibition as a function of proteoglycan concentration. We examined the inhibitory activity due to chondroitin sulfate, keratan sulfate, and N-linked oligosaccharides (**Figure 3**). We observed that chondroitinase ABC degradation of aggrecan on the plate surface enhances neurite outgrowth, as expected from the results of this and other laboratories. We obtained new data suggesting that removal of KS chains further enhances outgrowth. After degrading both chondroitin sulfate and keratan sulfate, we digested the substrate bound aggrecan with peptide-N-glycosidase F, which cleaves N-linked oligosaccharides from the core protein. This treatment resulted in a further enhancement of neurite outgrowth. These data are currently being prepared for publication.

We have performed neurite outgrowth assays to determine the effect of the different pools of astrocyte proteoglycans shown in Figure 1 upon neurite outgrowth (**Figure 4**). In this experiment we confirmed our previous result showing a dose-response relationship between substrate-bound aggrecan and neurite

outgrowth. We observed that the astroctye cell layer-derived PGs tend to enhance neurite outgrowth, an effect that become more pronounced in the later, more highly negatively charged fractions. We further observed that medium-derived proteoglycans tend to be inhibitory in the more highly negatively charged fractions. These data correlate with results of the sulfated disaccharide analysis, and the characterization of genetically distinct proteoglycans.

1b. Using a lentiviral system, we will induce primary astrocytes to degrade CSPGs via aggrecanase, chondroitinase, or both. Degradation of CSPGs will be confirmed using an anti-C-4-S antibody (2-B-6), which indentifies CSPG stubs following chondroitinase cleavage, or by antibodies to neoepitopes that are generated when aggrecanase cleaves the CSPG protein core into specific fragments. (months 5-7).

Cloning of ADAMTS-4 expression constructs. We generated three ADAMTS-4 expression constructs. The first plasmid (designated 701) contains the ADAMTS-4 coding sequence cloned into the vector pcDNA3.1/myc-his (-)A. Full sized (90 kDa pro-protein) ADAMTS-4 expressed from this plasmid, as well as the processed 68 kDa secreted form, can be detected with antibodies to ADAMTS-4, myc or His epitopes. The C-terminal His-tag enables purification of the protein on a nickel-chelating resin (i.e. ProBond). The second vector (designated 702) has an ADAMTS-4 insert lacking the N-terminal propeptide, with an N-terminal FLAG sequence adjacent to the first residue of the "processed" 68 kDa ADAMTS-4 sequence. This vector was constructed to determine if an N-terminal FLAG sequence would be more likely to persist in the expressed protein, since autocatalytic C-terminal proteolytic cleavage may remove a C-terminal FLAG sequence. A third construct (designated 703) places an ADAMTS-4 insert having a C-terminal FLAG sequence in the vector pcDNA3.1. The protein product can be affinity purified on a anti-FLAG affinity matrix, and can be detected on a Western blot using an anti-FLAG primary antibody. Each construct can be transiently or stably transfected into mammalian cells.

To produce recombinant ADAMTS-4 protein for experiments *in vitro*, or for injection into rat spinal cord injury sites (for Task 2a), we transiently transfected the 703 construct (having a C-terminal FLAG tag) into HEK293T cells for protein expression. This cell line was chosen because it is commonly used for high-level recombinant protein expression. In addition, we and others have found that the HEK293T cell line has characteristics of neuronal progenitor cells. We have found that this cell line produces proteoglycans characteristic of the CNS, including aggrecan, versican, decorin, neurocan, NG-2 and phosphacan. When transfected with the ADAMTS-4 expression plasmid, we found high levels of recombinant protein expression, and discovered that the cells themselves produce ADAMTS4 endogenously. The recombinant ADAMTS4 was found in both the cell layer and the medium. The enzyme in the medium, however, appeared to be in a complex with a high molecular weight material that is likely aggrecan or another CSPG. Examination of the western blot following colloidal

gold staining, to reveal all of the protein in each lane, showed a reduction in very high molecular weight protein in the cell lysate and the medium from ADAMTS4-transfected cells. This suggested that aggrecan, or other CSPGs produced by the HEK293 cells were actually being degraded by the expressed protease. Subsequently, another western blot analysis of lysate and medium from the same experiment showed the presence of ADAMTS4-generated aggrecan fragments, using an antibody to a specific neoepitope (anti-NITEGE) found on aggrecanase-generated aggrecan fragments. This fragment was seen in non-transfected lysates, apparently due to the endogenous ADAMTS4 activity, but was significantly increased in the medium from transfected cells. We regard this result as proof of principle for future experiments, which will be to express ADAMTS4 in glial scar to reduce the content of neurite outgrowth-inhibitory CSPGs. We purified recombinant ADAMTS4 from transfected HEK293 medium. Following transfection, HEK293 cells were incubated in medium containing heparin, to antagonize the strong association of ADAMTS-4 with PGs in the cell layer and medium. The enzyme was then purified using FLAG antibody-conjugated magnetic beads.

The pBOB/ADAMTS-4/FLAG construct lacks a reporter gene, so SDS-PAGE and Western blot analyses were used to screen for Flag tagged ADAMTS-4. The co-localization of ADAMTS-4 and Flag signal indicated successful transduction and expression of ADAMTS-4 (**Figure 5**). Further, chABC transduction was successfully done and is shown by a GFP reporter gene (green fluorescence) in a confluent astrocytes monolayer (**Figure 6**).

Preparation of pLVX-Tet-On Advanced, and pLVX-Tight-Pur-ADAMTS-4 vectors. We had the original goal of developing a lentiviral expression system that would enable the inducible expression of ADAMTS4 in primary cultured astrocytes, as well as inducible expression *in vivo*, in a rat model of spinal cord injury. However, this system was fraught with problems (some we overcame), but good practices dictated we abandon this direction. We used only the His-tagged and Flag-tagged expression constructs to accomplish our goals both *in vitro* and *in vivo*.

1c. Using the above system, we will determine if aggrecanase-mediated degradation of CSPGs in primary rat cortical astrocytes induces growth-inhibiting (CSPG-producing) astrocytes to become growth permissive (CSPG-degraded), and thereby foster regeneration of adult neurons (CST, RST, DC (DRG)) (months 8-11). We examined PG production and enzyme degradation of PGs in a co-culture model using transduced primary neurons and primary injured astrocytes. We cultured primary rat astrocytes as described previously with and without TGF-ß (5ng/ml, 48 H ; activates astrocytes and induces them to upregulate proteoglycans, i.e. simulates injury). Primary chicken DRG neurons (E9) were grown on confluent monolayers of these astrocytes, or in sparse cultures such that the neurons adhered to laminin or PLO first, then encountered

transfected astrocytes Figure 4 shows a representative image of DRG neurons and astrocytes in co-culture, demonstrating the basic paradigm. The co-cultures were then fixed and labeled with βIII-tubulin (TRITC) to image neurons, and GFAP (Cy5) 1° Ab's to image astrocytes, and DAPI to label all nuclei. (**Figure 7**; 40X). Quantitative analyses were performed, but are yet inconclusive. A larger sample size is required.

1d. We will test the responses of other neurons, e.g. 5HT, which have been shown to be robust following SCI in previous studies (months 12-13). Using the NS-1 neurite outgrowth assay, we will determine CSPG production (ELISA), CSPG cleavage (Western blot analyses), and aggrecanase activity (enzyme activity assays and immunostaining for neoepitopes) (Miwa, Gerken et al. 2006; Miwa, Gerken et al. 2006). Further, we will isolate aggrecanase-generated fragments and test their effect(s) on elongating axons *in vitro*.

For a variety of reasons, we limited our analyses throughout the study to only DRG neurons, the most relevant of the neuron types listed for this study, and the one to which we have the most previous data for comparison. We also tested NS-1 cells (see outgrowth assays above).

Aggrecanase-generated fragments were not tested *in vitro*, but are the focus of a renewed study.

Task 2. We will determine if aggrecanase-mediated degradation of CSPGs produced in vivo, in a rat spinal cord injury model system, will permit regeneration (months 13-36), and the effects of combining aggrecanase and chondroitinase treatments.

2a. Lentiviral transfection of ADAMTS-4 in an SCI model system will be performed, using currently approved methods (IACUC Protocol #2010-0702; approved 8-18-14). We will transduce, using a lentiviral vector, ADAMTS-4, within an injured region of the rat spinal cord (dorsal hemisection), (months 13-18).

Lentiviral preparation, and issues were discussed above.

Aggrecanase for studies in vivo. In preparation for injection *in vivo*, it was necessary to show that the His-tagged aggrecanase was active. We used both active and heat-inactivated aggrecanase for digestion of recombinant aggrecan, the results of which are shown in **Figure 8**. Recombinanat aggrecan (40ng) was digested in the absence (lane 1) or presence (lanes 2-5) of recombinant ADAMTS-4 (15 µl of 5mg/ml stock) at 37 °C. Incubation times ranged from 2 hours (lane 2), 12 hours (lane 3) to 24 hours (lanes 1, 4, 5). Recombinant ADAMTS-4 was heat-inactivated by heating at 95 °C for 30 minutes (lane 5). These data show

that the enzyme we were injecting *in vivo* following the dorsal hemisection spinal cord injury was active.

Surgical Procedures. All animal studies were conducted according to a University of Kentucky, IACUC-approved and DOD-approved protocol (#2010-0702). Adult male Sprague-Dawley rats (300-350g) were anesthetized with isoflurane (2-3% oxygen). Prior to the injury, a silastic tubing catheter (**Figure 9**) was placed in the intrathecal space through a small hole in the dura between the T1 and T2 vertebrae. The tubing was anchored to the T2 dorsal process and surrounding muscle via suture and Vetbond (3M), and was attached to an Azlet osmotic minipump containing pharmacological treatment, which was placed subcutaneously. The surgical procedure for the injury was initiated by performing a laminectomy of the caudal half of C5 and the rostral half of C7 vertebrae to expose the C6 and C7 dorsal roots. Using a Vibraknife (SM Onifer et al., 2005; RL Hill et al., 2009), a dorsal hemisection was performed at a depth of 1.35 mm (**Figure 10 and 11**). This injury produced a lesion that included the dorsal columns (sensory) and a portion of the corticospinal tracts (motor).

Pharmacological Treatments. Two pharmacological groups were used: Heat inactivated ADAMTS-4 (control) and active ADAMTS-4 (Figure 7). ADAMTS-4 was prepared by diluting the lyophilized protein into sterile saline at a concentration of 100nM. 200µL of the 100nM solution was loaded into each Azlet infusion mini pump at the time of surgery. Heat inactivated ADAMTS-4 was prepared by heating a 200µL aliquot of the 100nM solution to 95ºC for 30 minutes. The study was blinded. All pharmacological treatments were prepared by an individual who did not perform the surgery, and the tubes were labeled with a code unidentifiable to the assessor. A coin toss was used to determine the use of heat inactivated or activated ADAMTS-4. For behavioral assessments, see below.

Chondroitinase: A major goal of this study was to use combinational therapy (aggrecanase + chondroitinase (cABC)) to promote regeneration *in vivo*. One setback for this study was that our aggrecanase lentivirus was prepared using a different backbone than the chondroitinase lentiviral vector, supplied by the Smith lab (George Smith, PhD: colleague and previous collaborator). Taking advantage of the lentivirus expertise within SCoBIRC (Charles Mashburn, PhD), our ADAMTS-4 insert has also been cloned into a second construct, pCSC-SP-PW, which matches the construct used to generate the cABC lentivirus. This protocol will result in fewer experimental variables.

As we did for aggrecanase, we confirmed chondroitinase activity compared to a heat-inactivated control (**Figure 12**). We are just now ready for combinational studies *in vivo* with aggrecanase, but have reached the end of DOD funding. We have obtained foundation funding to finish this phase of the study.

2b. Aggrecan degradation in the lesion will be monitored with anti-neoepitope antibodies that will recognize aggrecan fragments. Using a variety of microscopy methods and established tract tracing techniques, neurons traversing the glial scar depleted of aggrecan by ADAMTS-4 will be quantified relative to untreated rats. (months 19-24).

Following 14 day infusion of aggrecanase, some animals were euthanized, and spinal cord tissue from test and control animals were screened via Western blot to confirm diffusion of ADAMTS-4 from the pump injection site. To identify aggrecanase distribution *in vivo*, we used Western blot analysis on spinal cord sections (**Figure 13**). Distribution of ADAMTS-4 in spinal cord tissue was visualized using a C-terminal His tag. A single spinal cord was cut into 7 equal sections representing the epicenter (E), caudal sections (C1 – C3) and rostral sections (R1 –R3) regions relative to the site of ADAMTS-4 injection (C6/7). The data show diffusion on aggrecanase into the spinal cord mainly at the epicenter and at least partially into both rostral and caudal directions.

To show that ADAMTS-4 was actively degrading aggrecan *in vivo*, we used a 1° antibody anti-NITEGE (to aggrecan core neoepitopes after aggrecanase treatment) (**Figure 14**, longitudinal sections; and **Figure 15,** transverse sections and high magnifications.) Sections were cut on a freezing microtome (cryostat) at 20 um, mounted on glass slides, and stained. In Figure 14, injured rats were infused with active ADAMTS-4 for 14 days. Brown reaction product (DAB) is staining with NITEGE antibody counterstained with Hematoxylin (R = Rostral, C = Caudal, and E = Epicenter.) Samples were selected in each direction from the injury site approximately 4 mm long and including a 4 mm section around the injury site (Dorsal = up, Ventral = down.) The catheter placement for the mini pump as at C2 (near cervical 6/7). In Figure 15, cross sections (transverse) were taken of rat spinal cord from sham and animals treated with ADAMTS-4 for 14 days, then stained with anti-NITEGE. Brown reaction product indicates aggrecan core protein neoepitope, thus cleaved by aggrecanase (both endogenous and exogenous aggrecan is degraded). Figure 15 = high magnification to show matrix degradation around individual cells at the white matter/gray matter interface of the dorsal cord. All data show that active enzyme was successfully delivered to the sites of injury.

2c. Histological assays to identify all cell types and molecules of interest in vivo. (months 25-28)

See 2b.

2d. Behavioral assays. Repeat in vivo paradigm and test behavioral recovery using the Reach, Grasp and Pellet Retrieval test (motor), the Grid Walking test (motor), and the Sticker Attention test (sensory) (months 29-36).

Two behavioral tests were performed to assess forelimb function after injury. All rats underwent acclimation and acquisition procedures prior to SCI.

Staircase Pellet Retrieval Test: Rats were tested three days a week specifically on Monday, Wednesday, and Friday. The day before testing food pellets were removed from the rat housing, leaving them without food for 18-24 hrs. Three times per week, rats were trained on the task for a total of 4 weeks. The first week of training rats were positioned above a trough filled with 45 mg dustless precision purified food pellets (Bio-Serv, Inc.) for 15 minutes each weekday for 1 week. The following week, rats retrieved pellets from the staircase apparatus for a 10 min session each weekday for 1 week. For the following two weeks, rats retrieved pellets during a 5-minute session. For the last three days of training, the number of pellets retrieved were recorded and used as a baseline measure for the analysis. Testing after SCI was similarly conducted (5 min. sessions) beginning 1 week after injury, once a week, and food was always removed the day prior to testing.

Sensory Sticker Test: Four weeks following injury, rats were subjected to the sensory sticker test. A 6 mm-diameter AVERY® self-adhesive color-coding sticker was applied to each forepaw palmar surface. Rats were then placed into a clean cage without bedding, and the time until the rat rotated the paw dorsally to expose the palmar surface to its mouth was measured. The maximum duration of each trial was 2 minutes. Five trials were conducted each with a 1-minute interval between trials. The attention times of the last 4 trials were averaged to assess sensory recovery.

Recombinant ADAMTS-4 cleaves aggrecan and enzymatic activity is lost upon heat inactivation. The results of the behavioral tests to date have been encouraging. The dorsal hemisection SCI at C6/7 in rat causes an impairment of the fine mechanical sensory systems and moderate motor impairment in the forepaw (due to limited penetration of Vibraknife into corticospinal tracts). Sensation was tested using the Sticker Sensory Task (**Figure 16**), and was done only after the final motor test, due to behavioral acclimation. Figure 16 shows there is a significant increase in the time an injured rat first notices the sticker placed on its paw. However, treatment with a recombinant aggrecanase abolished this increase and returned it to an interval not statistically different from uninjured animals. Figure 16 shows that over a course of 5 trials, the injured animals (both those receiving active aggrecanase and those not) showed reduced latency though not returning to uninjured sham levels. However, a trend suggested that aggrecanase treatment reduces the amount of time till first notice in the initial trials, likely before the animals get acclimated to the test.

Figure 17 shows results of the Staircase Retrieval task, an indication of forepaw use for reaching, grasping and retrieving food to the endpoint of successful consumption of food pellets. These data show significant improvement in the Staircase Retrieval test with 14-day aggrecanase administration. Future studies using aggrecanase and chondroitinase combined will determine if the two enzymes together show synergistic results, above that for just aggrecanase alone.

Personnel:

Personnel in addition to the PI who contributed to the studies, either throughout the project, or for some portion of it were: Dr. Thomas Hering, PhD., Case Western Reserve University, Cleveland, OH; Stephen Onifer, PhD, now at Palmer Rehabilitation Center in Davenport, IA; George M. Smith, PhD, Drexel University; Adrian Centers, MS, Regenerex, Louisville, KY.

FIGURES

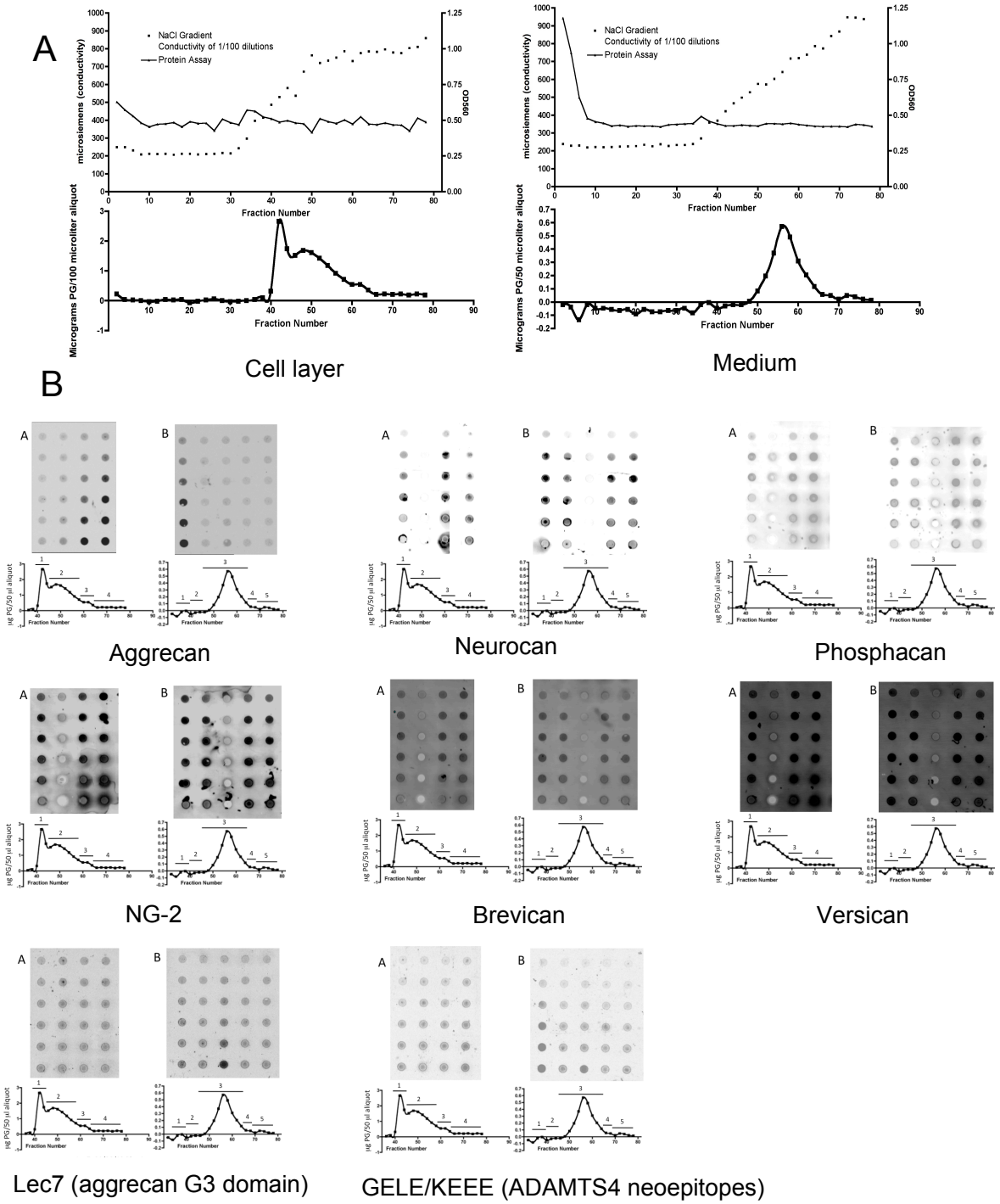

Figure 1. Analysis of proteoglycans produced by activated rat cortical astrocytes. (A) Proteoglycans were purified by size exclusion and ion exchange chromatography. DEAE chromatographic profiles are shown for astrocyte cell layer (A) and medium (B) eluted with a 0.15 to 1.0 M NaCl gradient. (B) Fractions were pooled, concentrated and characterized by dot blot analysis using specific antibodies to different proteoglycans. The dot blot analysis above the chomatogram shows reactivity of serial dilutions of pooled fractions with an antibody to the G1 domain of aggrecan.

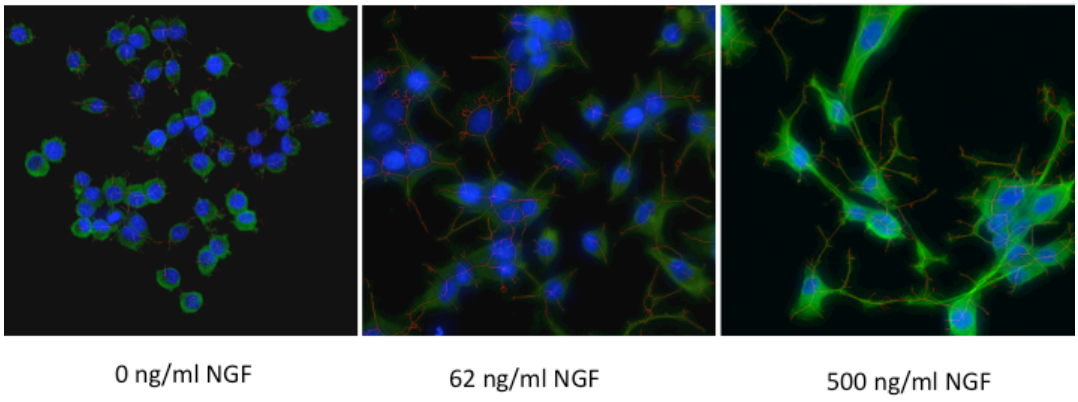

Figure 2. Neurite outgrowth assay: Response of NS-1 cells to NGF treatment. Following culture of NS-1 cells with NGF on proteoglycan-coated substratum, cells were imaged and neurite outgrowth was measured using the Neurite Tracer Image J plugin.

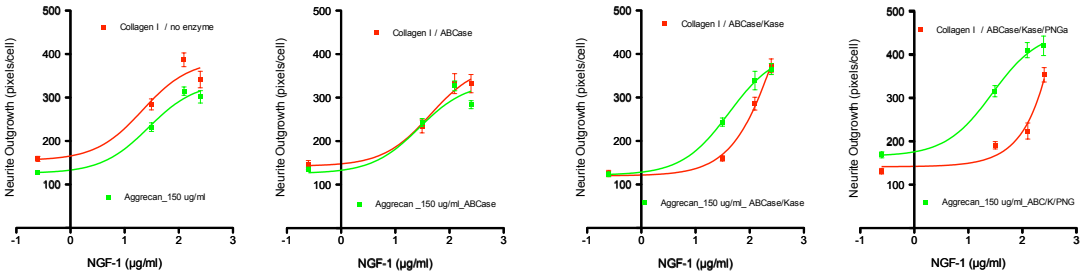

Figure 3. Neurite outgrowth assay: Effect of glycohydrolase treatment of aggrecan-coated substratum. NS-1 cells were grown on collagen I coated surfaces (red) or on surfaces treated sequentially with either chondroitinase ABC alone (ABCase) chondroitinase ABC and endo-beta-glycosidase to degrade keratan sulfate (K'ase), or ABCase, K'ase, and peptide N-glycosidase F (PNGase).

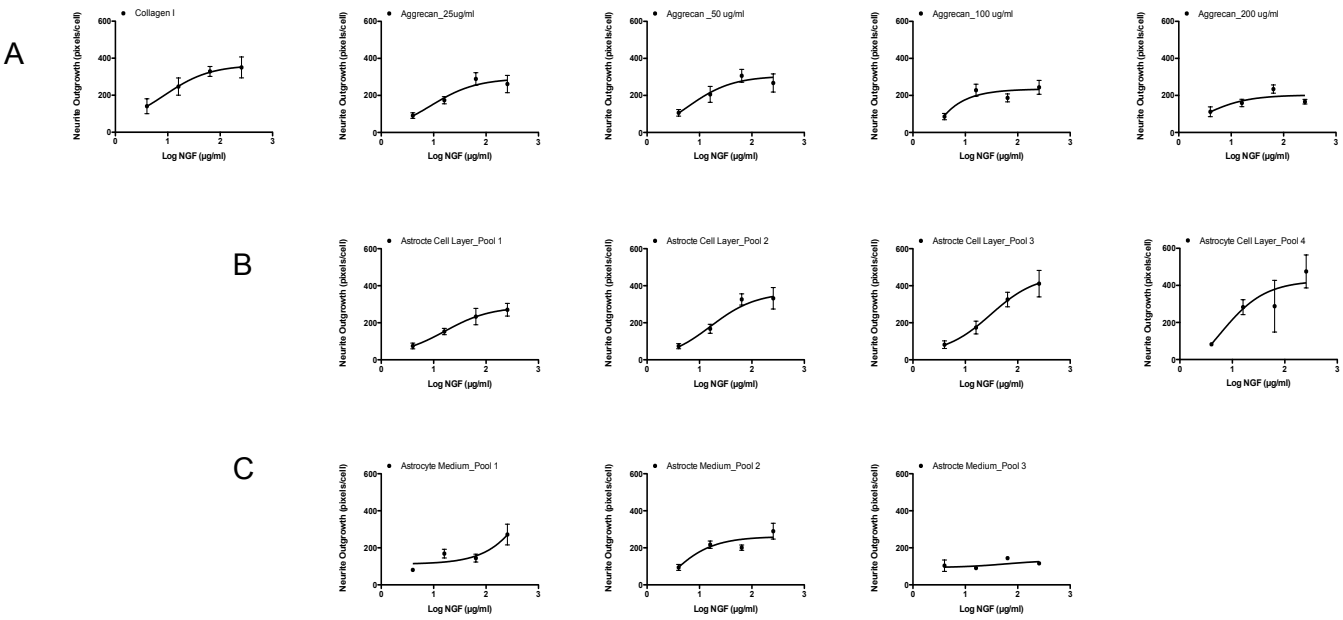

Figure 4. Neurite outgrowth assay: Effect of activated rat cortical astrocyte proteoglycans. (A) bovine cartilage aggrecan control series. Increasing concentrations of aggrecan applied to the culture dish prior to plating and outgrowth of NS-1 cells. (B) Astrocyte cell layer proteoglycan pools (pool #1 to pool #4) from DEAE Sepharose fractionated PGs were applied to the culture dish. Equivalent amounts of GAG from each pool (as determined by uronic acid) were applied. (C) Astrocyte medium proteoglycan pools (pool #1 to pool #3) from DEAE Sepharose fractionated PGs were applied to the culture dish,

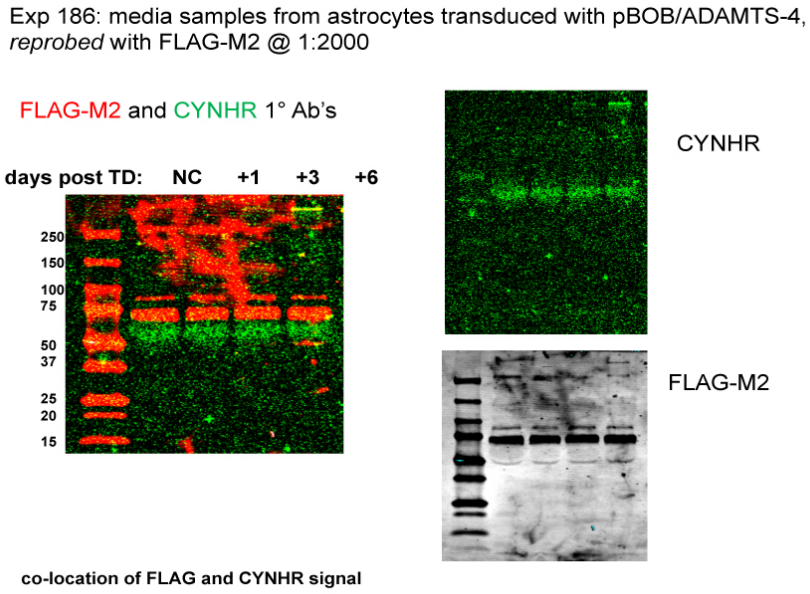

Figure 5. Media samples from astrocytes transduced with pBOB/ADAMTS-4, probed with FLAG-M2 @ 1:2000. Co-localization of ADAMTS-4 and Flag signal indicate successful transduction and expression of ADAMTS-4

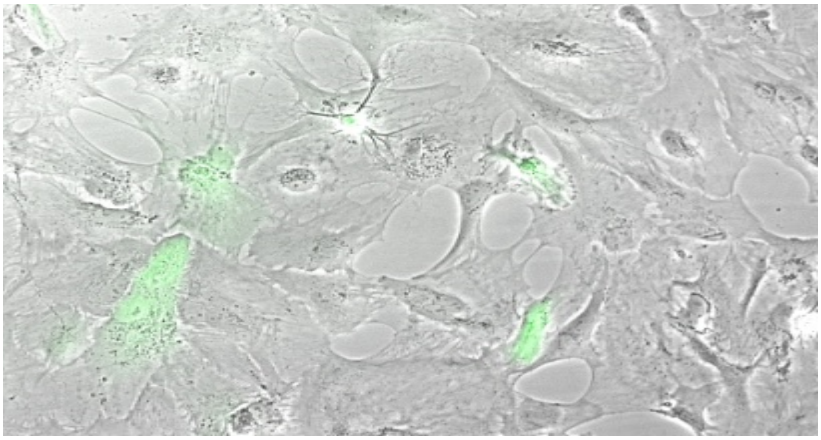

Figure 6. Rat primary astrocytes transduced with pBOB/chABC/GFP lentivirus

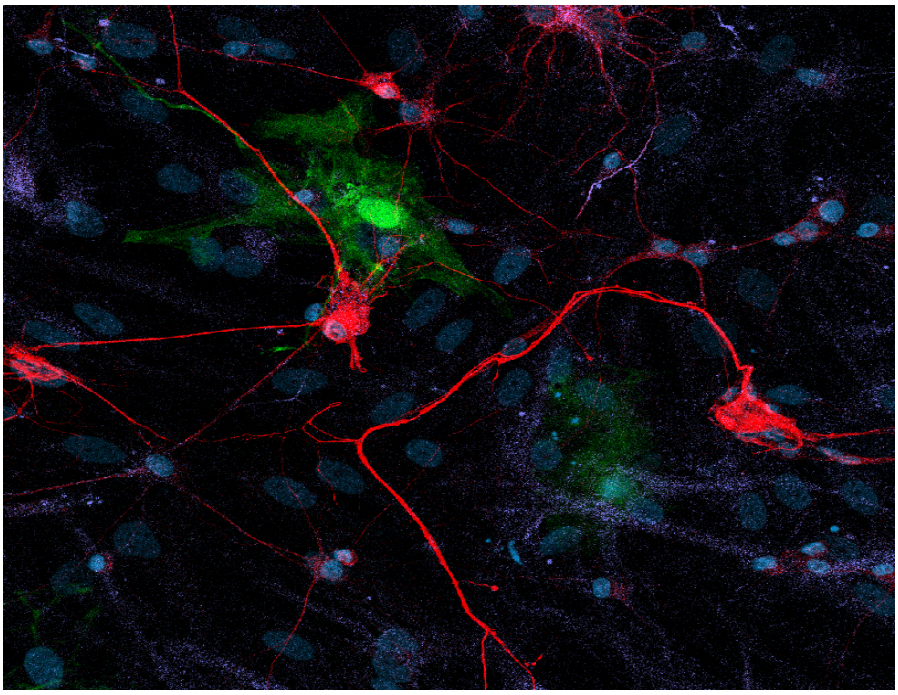

Figure 7. Representative image of DRG neurons and astrocytes in co-culture, demonstrating the basic paradigm. Rat primary astrocytes are cultured in the presence of TGFβ (5ng/ml, 48 H), then transformed overnight (to over or under express a variety of CSPGs), followed by seeding of chick DRG neurons (E9). The co-cultures are then fixed and labeled with βIII-tubulin (TRITC) to image neurons, and GFAP (Cy5) 1° Ab's to image astrocutes, and DAPI to abel all nuclei. (40X). Quantitative analyses are ongoing.

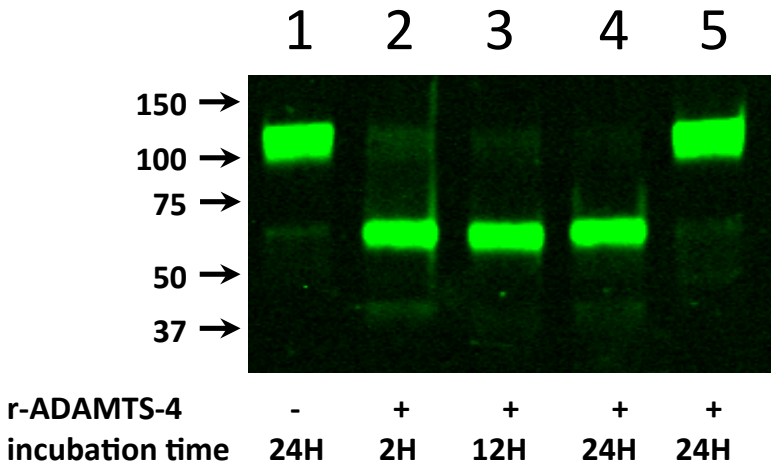

Figure 8. Active versus Heat Inactivated ADAMTS-4 digestion of aggrecan. Aggrecan (40ng) was digested in the absence (lane 1) or presence (lane 2-5) of ADAMTS-4 (60ug) at 37°C. Incubation ranged from 2 hours (lane 2), 12 hours (lane 3) or 24 hours (lanes 1,4,5). ADAMTS-4 was heat-inactivated by heating at 95°C for 30 minutes (lane 5).

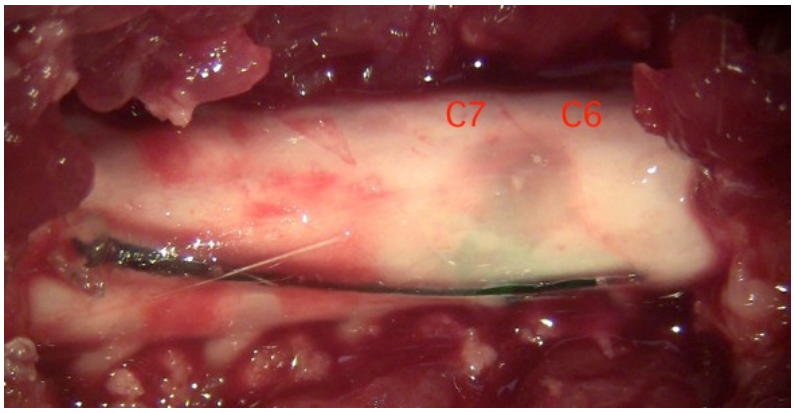

Figure 9. Osmotic mini pump placed at T1 (not shown) and threaded under the dura to administer enzyme at C6/C7. Rates and volumes are described in text.

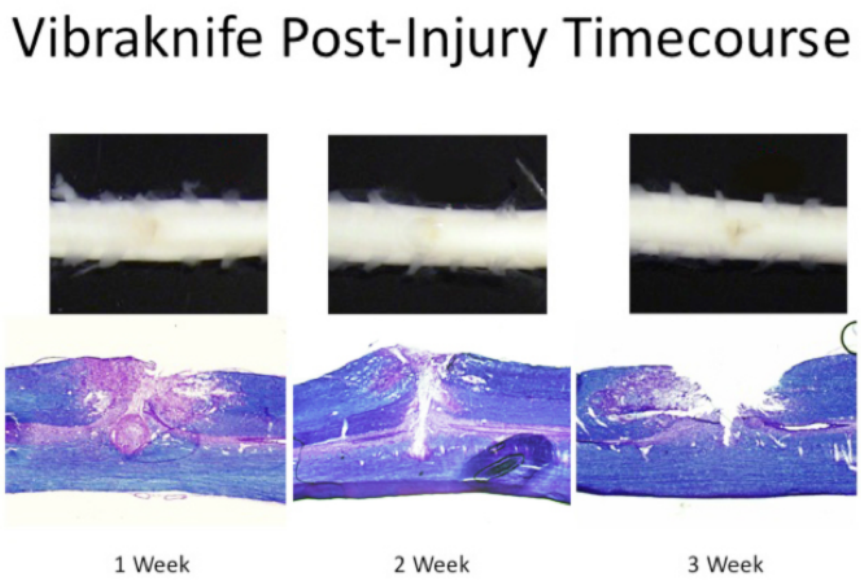

Figure 10. Post injury histology of Vibraknife injured rat spinal cord at 1, 2, and 3 weeks post injury. Longitudinal sections taken at or near midline, n=6.

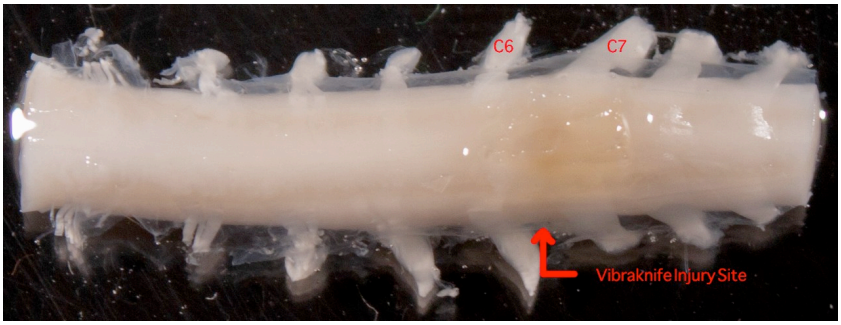

Figure 11. Vibraknife injury site between vertebrae C6 and C7.

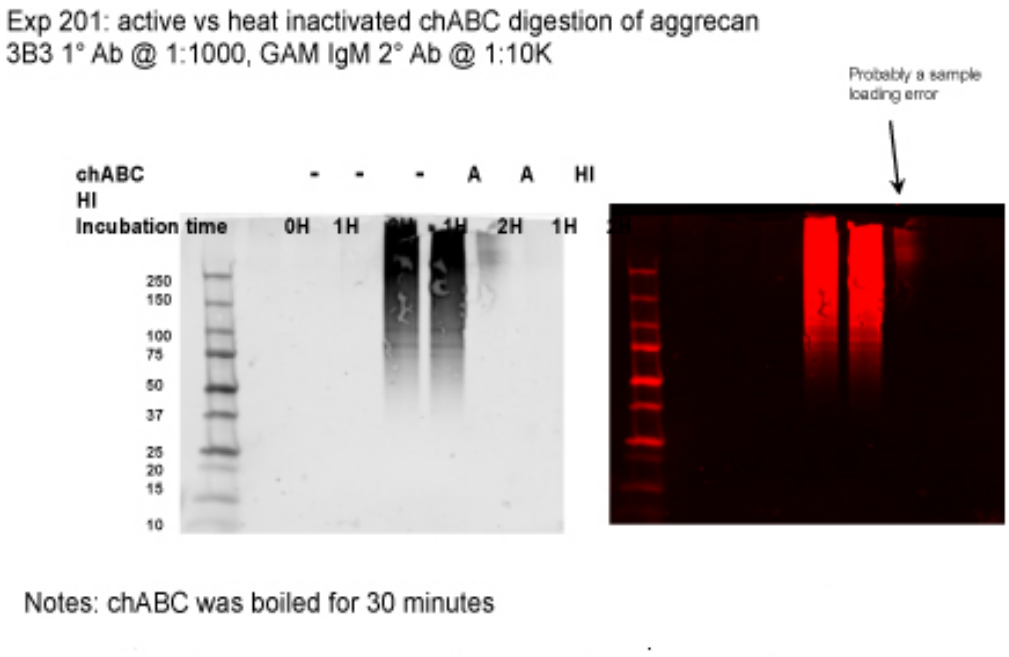

Figure 12. Chondroitinase ABC activity assay. Degradation confirmed using 1° Ab clone 3-B-3 to C-6-S stubs revealed following enzyme degradation.

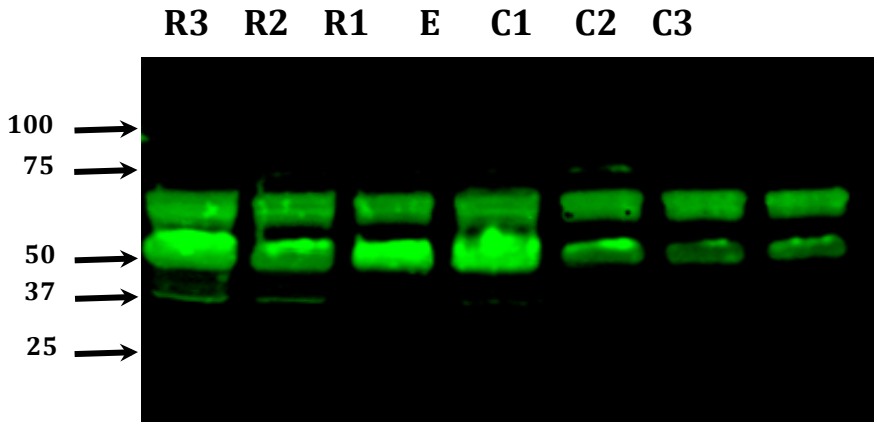

Figure 13. Distribution of recombinant ADAMTS-4 in spinal cord tissue as visualized by a C-terminal His tag. A single spinal cord section has been cut into 7 equal sections representing the epicenter (E), caudal (C1 – C3) and rostral (R1 –R3) regions relative to the site of r-ADAMTS-4 injection

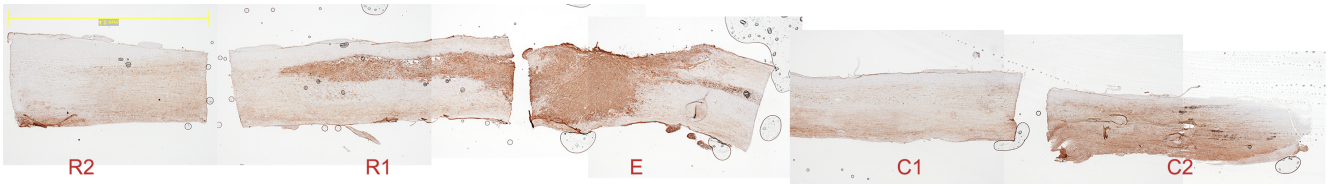

Figure 14. Injured rat infused with ACTIVE ADAMTS-4 for 14 days. Brown reaction product indicates NITEGE reactivity to ADAMTS4 induced neoepitopes; counterstained with Hematoxylin. R = Rostral, C = Caudal, and E = Epicenter. Two sections in each direction from the injury site approximately 4 mm long and including a 4 mm section around the injury site.

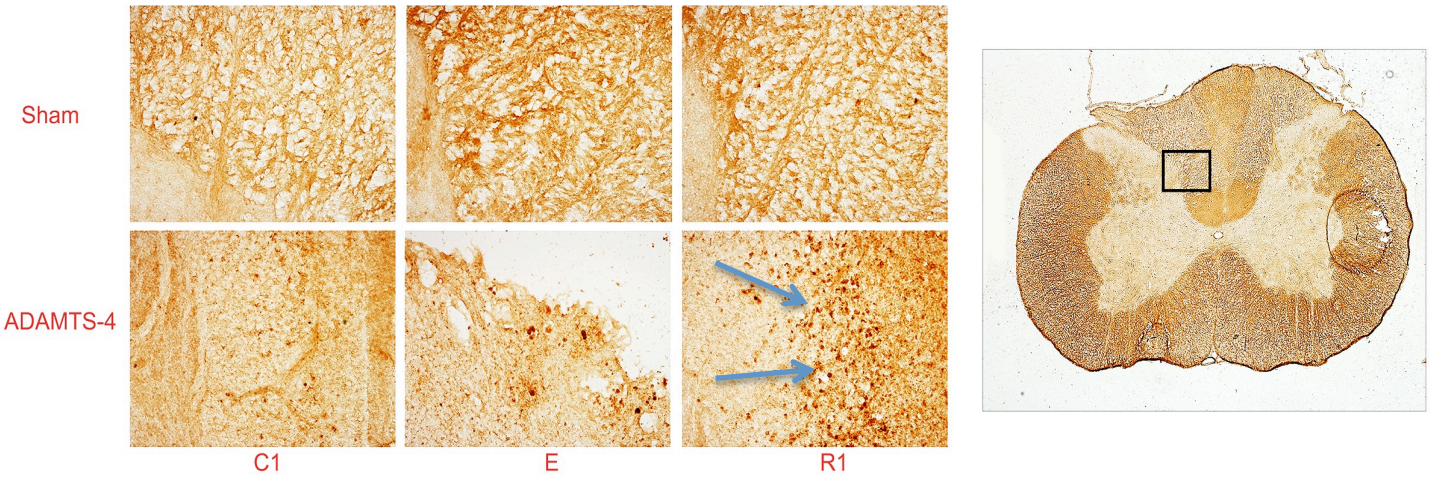

Figure 15. High magnification of images; representative cross section shown at right. Note the intense reaction product, i.e. degradation of aggrecan (blue arrows) surrounding individual cells at the white matter/gray matter interface. High mags are taken at the dorsal horn of the spinal cord (box in right image). Caudal to injury, C1; Epicenter, E; and Rostral to injury, R1.

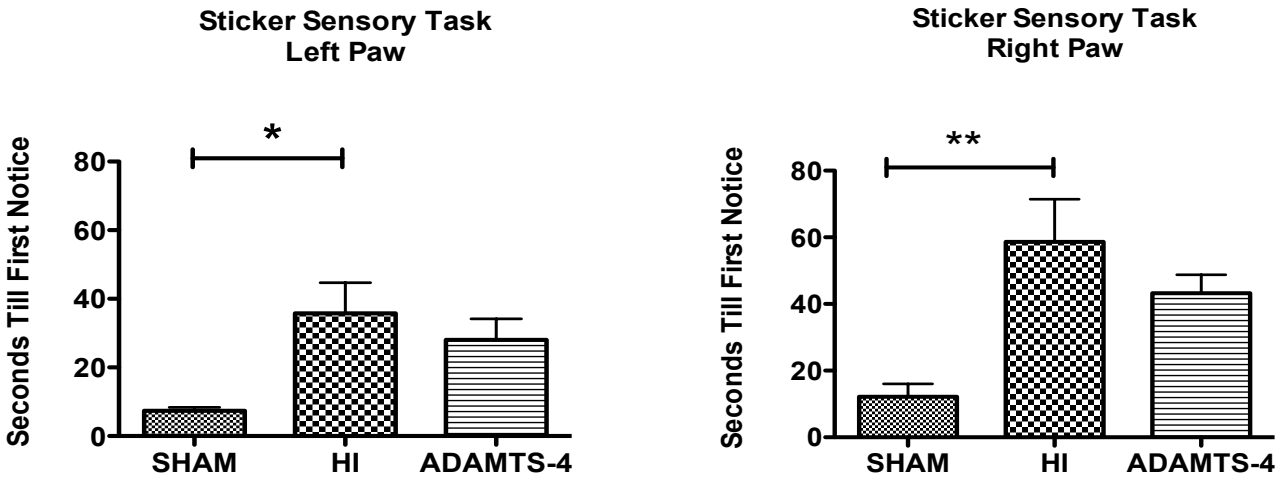

Figure 16. Treatment with active ADAMTS-4 abolishes significant deficiency in right and left paw tactile sense following dorsal column hemisection. Twenty-eight (28) days post-injury, rats were subjected to the sticker sensory task. Dorsal hemisection produced a deficiency in the time until the rat first noticed the sticker on both the left and right paw. Treatment with active ADAMTS-4 abolished this significant difference. * p<0.05, ** p<0.001 using Bonferroni post-hoc analysis.

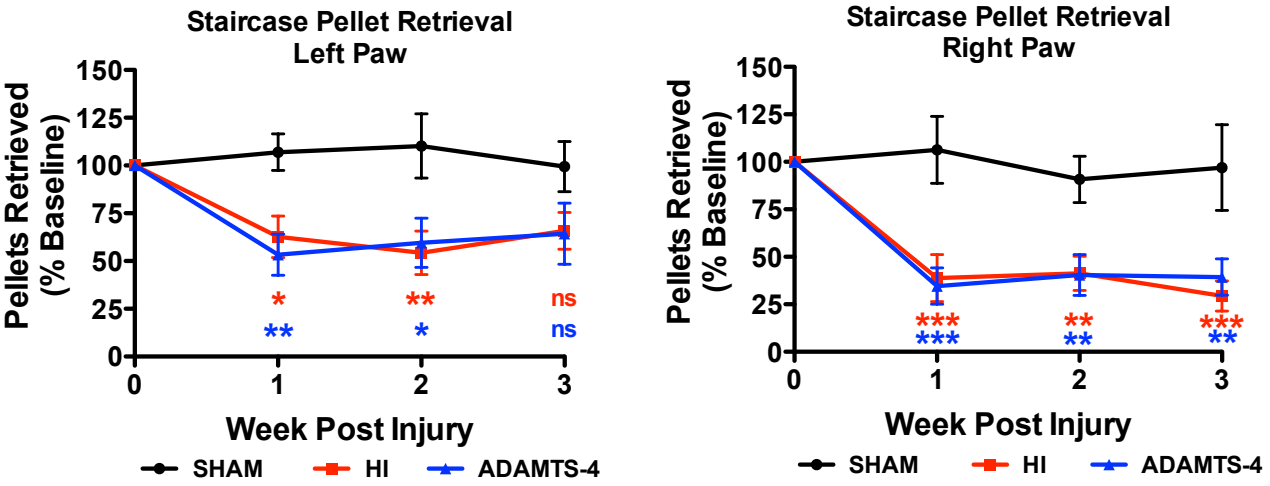

Figure 17. Treatment with active ADAMTS-4 had no effect on performance during the staircase pellet retrieval task following dorsal hemisection. Following dorsal hemisection, there was a significant decrease in the number of pellets retrieved at week 1 and 2 of the left paw and at week 1,2, and 3 of the right paw, indicating a grasp and retrieval deficit, likely due to the injury of the CST and RST. Treatment with active ADAMTS-4 did not significantly improve this performance. Data was analyzed using a two-way ANOVA (by Treatment and Time) followed by Bonferroni post-hoc analysis. * p<0.05, ** p<0.01, *** p<0.001, ns - no significant difference.

Key research accomplishments

- Removal of KS chains further enhanced NS-1 outgrowth, beyond that of chondroitinase treatment alone

- Further degradation with peptide-N-glycosidase F, which cleaves N-linked oligosaccharides from the core protein, resulted in a further enhancement of NS-1 neurite outgrowth

- Establishment of a high-throughput technique for assessing the effects of HEK293T cell (glial like) chondroitin sulfate proteoglycans.

- Successful diffusion of recombinant aggrecanase into spinal cord tissue at the site of injury and assessment of resulting neurite behavior

- Aggrecanase treatment reduces the severity of motor and sensory deficits following C6/7 spinal cord (dorsal hemisection) injury.

Reportable outcomes.

I. Abstracts

Transient expression and purification of aggrecanase (adamts-4) from hek293t cells. Jonathan Davies, Diane M. Snow*, and T. M. Hering*. The University of Kentucky, Spinal Cord and Brain Injury Research Center, Lexington, KY 40536.

Beller, JA, Hering, TM and **Snow, DM**. Biosynthesis of chondroitin sulfate proteoglycans in HEK293T cells. KSCHIRT Symposium, May 2011, Louisville, KY.

Beller, JA, Hering, TM, and **Snow, DM**. Biosynthesis of Chondroitin Sulfate Proteoglycans in Human Embryonic Kidney Cells. 29th Natl Neurotrauma Soc Symp, Ft. Lauderdale, FL; June. 10-14, 2011.

Hering, TM, Davies, J., **Snow, DM**. Transient expression and purification of aggrecanase (ADAMTS-4) from HEK293T cells. 14th International Symposiuim for Neural Regeneration, December 7-11, 2011, Monterey, CA.

Beller, JA, Hering, TM, and **Snow, DM**. HEK293T cells produce chondroitin sulfate proteoglycans with varied sulfation patterns, express multiple carbohydrate sulfotransferases, and are a novel system for the production of "Designer PGs". 14th International Symposiuim for Neural Regeneration, December 7-11, 2011, Monterey, CA.

Beller JA, Calulot CM, Hering TM, **Snow DM**, 2012. Post-translational modifications of aggrecan and inhibition of neurite outgrowth. Journal of Neurotrauma (Natl Neurotrauma Soc. meeting), July 2012.

Beller, JA, Hering, TM, Calulot, CM, and Snow, DM. A Novel High-Throughput Neurite Outgrowth Assay: Enzymatic Digestion Reveals Contribution of Post-Translational Modifications of Aggrecan on Neurite Inhibition. 19th Annual Kentucky Spinal Cord & Head Injury Research Trust Symposium, Louisville, Kentucky, May 6, 2013.

Beller JA, Hering TM, Calulot CM, and **Snow DM**, 2013. A Novel High-throughput Neurite Outgrowth Assay: Enzymatic Digestion Reveals Contributions of Post-translational Modifications of Aggrecan on Neurite Inhibition. Proceedings J. Neurotrauma (Natl Neurotrauma Soc. meeting), July 2013, Nashville, TN.

Beller, JA; Calulot, CM; Centers,AP; Hering, TM; and **Snow, DM. (**2013). The aggrecan-degrading enzyme, ADAMTS-4, enhances functional recovery of front paw tactile sensation using a cervical spinal cord dorsal hemisection injury model. International Society for Neural Regeneration, Pacific Grove, CA, Dec. 11-14, 2013.

II. Summer Research Grants

Jonathan Davies, undergraduate summer research grant from the Appalachian & Minority Science, Technology, Engineering and Mathematics Majors (AMSTEMM) program. *(this grant allowed Mr. Davies to work 20 hr/wk on the project during the summer – June-Aug, 2011).*

III. Scholarships

Jonathan Davies, undergraduate scholarship from the Appalachian & Minority Science, Technology, Engineering and Mathematics Majors (AMSTEMM) program. *(this scholarship allowed Mr. Davies to build on the techniques he learned and the data he acquired during the summer of 2011; he is currently in my lab and plans to continue through summer 2012).*

IV. Manuscripts

Beller, JA, and **Snow, DM**. (2014). Proteoglycans: Road Signs for Neurite Outgrowth. *In: Neuronal Growth Cones and Regeneration: Gridlock within the Extracellular Matrix*. *Ed. D. Snow* – Neural Regeneration Research. Feb, 2014.

Beller, JA, Hering, TM, and **Snow, DM**. (2014) Techniques for examining the effect of substratum-bound proteoglycans on neurite outgrowth *in vitro*. In: JB Leach and EM Powell (eds) Neuromethods: Extracellular Matrix. Humana Press, New York.

Beller, JA, Hering, TM, and **Snow, DM.** (2014) *High-throughput quantitative assay for analyzing neurite outgrowth on a uniform substratum: the cell-substratum assay*. In: JB Leach and EM Powell (eds) Neuromethods: Extracellular Matrix. Humana Press, New York.

Snow, DM., 'Designer molecules', International Innovation, Issue 123: Health partnership, January 2014, Research Media, UK, pp 88-90; ISSN 2051-8552.

Snow, DM; Beller, JA; Calulot, CM; Centers, AP; Hering, TM**.** The aggrecan-degrading enzyme, ADAMTS-4, enhances functional recovery of front paw tactile sensation using a cervical spinal cord dorsal hemisection injury model. *(In preparation for J. Neurotrauma)*

Conclusions

Importance of implications of completed research: We have generated the specific tools and methodologies necessary to test aggrecanase and chondroitinase *in vitro* and *in vivo*. We have done studies to optimize the *in vivo* model and enzyme delivery methods. We have introduced aggrecanase *in vivo* using a dorsal hemisection injury model in the rodent and have processed spinal cord tissues to examine cellular and molecular changes. We have assessed motor and sensory deficits following injury without aggrecanse treatments (controls) and have shown that treatment with aggrecanase can partially alleviate these deficits.

Recommended changes: No recommended changes.

"So what?" *(evaluate knowledge gained as a scientific or medical product*): No scientific or medical product was developed. However, the present data open the doors for further research to develop aggrecanase as a potential therapy for SCI. The combinational use of aggrecanase with chondroitinase may have therapeutic potential, but this condition has not yet been substantiated by the data.

References

1. Fawcett JW, Asher RA: **The glial scar and central nervous system repair**. *Brain Res Bull* 1999, **49**(6):377-391.
2. Silver J, Miller JH: **Regeneration beyond the glial scar**. *Nat Rev Neurosci* 2004, **5**:146-156.
3. Faulkner J, Herrmann JE, Woo MJ, Tansey KE, Doan NB, MV S: **Reactive astrocytes protect tissue and preserve function after spinal cord injury.** *J Neurosci* 2004, **24**(9):2143-2155.
4. Myer D, Gurkoff G, Lee S, Hovda D, Sofroniew M: **Essential protective roles of reactive astrocytes in traumatic brain injury.** *Brain* 2006, **129**(10):2761-2772.
5. Heinegard D, Paulsson M: **Structure and metabolism of proteoglycans.** In: *Extracellular Matrix Biochemistry.* Edited by Piez KA, Reddi AH. New York. Amsterdam. Oxford.: Elsevier; 1984: 277-382.
6. Jones L, Margolis R, Tuszynski M: **The chondroitin sulfate proteoglycans neurocan, brevican, phosphacan, and versican are differentially regulated following spinal cord injury.** *Experimental Neurology* 2003, **182** 399–411.
7. Morgenstern DA, Asher RA, Fawcett J: **Chondroitin sulphate proteoglycans in the CNS injury response**. *Progress in Brain Research* 2002, **137**:313-332.
8. Bovolenta P, Wandosell F, Nieto-Sampedro M: **CNS glial scar tissue: a source of molecules which inhibit central neurite outgrowth.** In: *Progress in Brain Research.* Edited by A.C.H. Yu LH, M.D. Norenberg, E. Sykova and S.G. Waxman, vol. 94: Elsevier Science Publishers, B.V.; 1992: 367-379.
9. Snow DM, Mullins N, Hynds DL: **Nervous system-derived chondroitin sulfate proteoglycans regulate growth cone morphology and inhibit neurite outgrowth: a light, epifluorescence, and electron microscopy study.** *Micr Res Tech* 2001, **54**:273-286.
10. Laabs T, Wang H, Katagiri Y, McCann T, Fawcett J, Geller H: **Inhibiting glycosaminoglycan chain polymerization decreases the inhibitory activity of astrocyte-derived chondroitin sulfate proteoglycans.** *J Neurosci* 2007, **27**(52):14494-14501.
11. Clement AM, Nadanaka S, Masayama K, Mandl C, Sugahara K, Faissner A: **The DSD-1 carbohydrate epitope depends on sulfation, correlates with chondroitin sulfate D motifs, and is sufficient to promote neurite outgrowth**. *J Biol Chem* 1998, **273**:28444-28453.
12. Faissner A, Clement A, Lochter A, Streit A, Mandl C, Schachner M: **Isolation of a neural chondroitin sulfate proteoglycan with neurite outgrowth promoting properties.** *J Cell Biol* 1994, **126**:783-799.
13. Gilbert RJ, McKeon RJ, Darr A, Calabro A, Hascall VC, Bellamkonda RV: **CS-4,6 is differentially upregulated in glial scar and is a potent inhibitor of neurite extension**. *Mol Cell Neurosci* 2005, **29**(4):545-558.
14. Snow DM, Lemmon V, Carrino DA, Caplan AI, Silver J: **Sulfated proteoglycans in astroglial barriers inhibit neurite outgrowth in vitro.** *Exp Neurol* 1990, **109**:111-130.
15. Moon LDF, Asher RA, Rhodes K, Fawcett J: **Regeneration of CNS axons back to their target following treatment of adult rat brain with chondroitinase ABC**. *Nature Neuroscience* 2001, **4**(5):465-466.
16. Bradbury EJ, Moon LDF, Popat RJ, King VR, Bennett GS, Patel PN, Fawcett J, McMahon SB: **Chondroitinase ABC promotes funcitonal recovery after spinal cord injury**. *Nature* 2002, **416**:636-640.
17. Tester NJ, Plaas AH, Howland DR: **Motor recovery following chondroitinase ABC application to the acutely injured cat spinal cord.** 2004, **231.9**(2004 Abstract Viewer/Itinerary Planner.Washington, DC: Society for Neuroscience).
18. Houle J, Tom V, Mayes D, Wagoner G, Phillips N, Silver J: **Combining an Autologous Peripheral Nervous System "Bridge" and Matrix Modification by Chondroitinase Allows Robust, Functional Regeneration beyond a Hemisection Lesion of the Adult Rat Spinal Cord**. *J Neurosci* 2006, **26**(28):7405–7415
19. Curinga GM, Snow DM, Mashburn C, Kohler K, Thobaben R, Caggiano A, Smith G: **Mammalian-produced chondroitinase AC mitigates axon inhibition by chondroitin sulfate proteoglycans.** *J Neurochem* 2007, **102**:275-288.

20. Massey JM, Hubscher CH, Wagoner MR, Decker JA, Amps J, Silver J, Onifer SM: **Chondroitinase ABC digestion of the perineuronal net promotes functional collateral sprouting in the cuneate nucleus after cervical spinal cord injury**. *J Neurosci* 2006, **26**(16):4406-4414.
21. Tester NJ, Howland DR: **Chondroitinase ABC improves basic and skilled locomotion in spinal cord injured cats**. *Exp Neurol* 2008, **209**(2):483-496.
22. Tom VJ, Kadakia R, Santi L, Houle JD: **Administration of Chondroitinase ABC Rostral or Caudal to a Spinal Cord Injury Site Promotes Anatomical But Not Functional Plasticity**. *J Neurotrauma* 2009.
23. Tom VJ, Sandrow-Feinberg HR, Miller K, Santi L, Connors T, Lemay MA, Houle JD: **Combining peripheral nerve grafts and chondroitinase promotes functional axonal regeneration in the chronically injured spinal cord**. *J Neurosci* 2009, **29**(47):14881-14890.
24. Garcia-Alias G, Barkhuysen S, Buckle M, Fawcett JW: **Chondroitinase ABC treatment opens a window of opportunity for task-specific rehabilitation**. *Nat Neurosci* 2009, **12**(9):1145-1151.
25. Tortella M, Burn T, Pratta M, Abbaszade I, Hollis J, Liu R, Rosenfield S, Copeland R, Decicco C, Wynn R *et al*: **Purification, and cloning of aggrecanase-1: a member of the ADAMTS family of proteins.** *Science* 1999, **284**:1664-1666.
26. Nagase H: **Aggrecanase in cartilage matrix breakdown.** *Connective Tissue* 2001, **33**:27-32.
27. Miwa H, Gerken T, Hering T: **Effects of covalently attached chondroitin sulfate on aggrecan cleavage by ADAMTS-4 and MMP-13.** *Matrix Biol* 2006, **[Epub ahead of print]**.
28. Miwa HE, Gerken TA, Hering TM: **The susceptibility of chondroitin sulfate-free recombinant aggrecan to ADAMTS-4.** In: *Trans Orthop Res Soc: 2006; Chicago, IL*; 2006: 1458.
29. Ajmo JM, Eakin AK, Hamel MG, Gottschall PE: **Discordant localization of WFA reactivity and brevican/ADAMTS-derived fragment in rodent brain**. *BMC Neurosci* 2008, **9**:14.
30. Hamel MG, Ajmo JM, Leonardo CC, Zuo F, Sandy JD, Gottschall PE: **Multimodal signaling by the ADAMTSs (a disintegrin and metalloproteinase with thrombospondin motifs) promotes neurite extension**. *Exp Neurol* 2008, **210**(2):428-440.
31. Mayer J, Hamel MG, Gottschall PE: **Evidence for proteolytic cleavage of brevican by the ADAMTSs in the dentate gyrus after excitotoxic lesion of the mouse entorhinal cortex**. *BMC Neurosci* 2005, **6**:52.
32. Westling J, Gottschall P, Thompson V, Cockburn A, Perides G, Zimmermann D, Sandy J: **ADAMTS4 (aggrecanase-1) cleaves human brain versican V2 at Glu405-Gln406 to generate glial hyaluronate binding protein.** *Biochem J* 2004, **377**(3):787-795.
33. Massey JM, Amps J, Viapiano MS, Matthews RT, Wagoner MR, Whitaker CM, Alilain W, Yonkof AL, Khalyfa A, Cooper NG *et al*: **Increased chondroitin sulfate proteoglycan expression in denervated brainstem targets following spinal cord injury creates a barrier to axonal regeneration overcome by chondroitinase ABC and neurotrophin-3**. *Exp Neurol* 2008, **209**(2):426-445.
34. Lemons ML, Sandy JD, Anderson DK, Howland DR: **Intact aggrecan and fragments generated by both aggrecanse and metalloproteinase-like activites are present in the developing and adult rat spinal cord and their relative abundance is altered by injury**. *J Neurosci* 2001, **21**(13):4772-4781.
35. Andrews EM, Yin QFF, Viapiano MS, Jakeman LB: **Alterations in chondroitin sulfate proteoglycan expression both at and distal to the site of spinal cord injury in rats.** *Soc for Neurosci Abstr* 2009, **563.10**.
36. Nagase H, Kashiwagi M: **Aggrecanases and cartilage matrix degradation.** *Arthritis Res Ther* 2003, **5**:94-103.
37. Murakami T, Su W, Ohtsuka A, Abe K, Ninomiya Y: **Perineuronal nets of proteoglycans in the adult mouse brain are digested by collagenase.** *Arch Histol Cytol* 1999, **62** 199-204.
38. Sasaki M, Seo-Kiryu S, Kato R, Kita S, Kiyama H: **A disintegrin and metalloprotease with thrombospondin type1 motifs (ADAMTS-1) and IL-1 receptor type 1 mRNAs are simultaneously induced in nerve injured motor neurons.** *Brain Res Mol Brain Res* 2001, **89**:158-163.

39. Satoh KA, Suzuki N, Yokota H: **ADAMTS-4 (a disintegrin and metalloproteinase with thrombospondin motifs) is transcriptionally induced in beta-amyloid treated rat astrocytes.** *Neurosci Lett* 2000, **289** 177-180.
40. Yuan W, Matthews RT, Sandy JD, Gottschall PE: **Association between protease-specific proteolytic cleavage of brevican and synaptic loss in the dentate gyrus of kainate-treated rats.** *Neuroscience* 2002, **114**:1091-1101.
41. Curinga G, Jin Y, Zhang C, Mashburn C, Snow D, Smith G: **Neurons transduced with engineered chondroitinase demonstrate enhanced outgrowth.** . In preparation.
42. Onifer SM, Zhang YP, Burke DA, Brooks DL, Decker JA, McClure NJ, Floyd AR, Hall J, Proffitt BL, Shields CB *et al*: **Adult rat forelimb dysfunction after dorsal cervical spinal cord injury**. *Exp Neurol* 2005, **192**(1):25-38.
43. Curinga G, Jin Y, Zhang C, Mashburn C, Snow D, G. S: **Engineering chondroitinase to enhance axon regeneration through the glial scar.** *38th Ann Soc Neurosci Abst, Washington, DC* 2008.
44. Curinga G, Jin Y, Zhang C, Mashburn C, Snow D, Smith G: **Chase-ing regeneration: engineering chondroitinase for mammalian expression.** *Exper Biol Abst, New Orleans, LA* 2009.
45. Smith GM, Strunz C: **Growth factor and cytokine regulation of chondroitin sulfate proteoglycans by astrocytes.** *Glia* 2005, **52**(3):209-218.
46. Sarmiere PD, Weigle CM, Tamkun MM: **The Kv2.1 K+ channel targets to the axon initial segment of hippocampal and cortical neurons in culture and in situ**. *BMC Neurosci* 2008, **9**:112.
47. Onifer SM, Rodríguez JF, Benitez JC, Hesse DH, Kim DT, Pacheco JT, Perrone JV, Santiago DI, A. M-A: **Cervical spinal cord injury in the adult rat: Forelimb function after intraspinal transplantation of trophic factor-treated fetal spinal cord cells.** *Soc Neurosci Abstr* 1994, **20**:1706.
48. Lemons ML, Sandy JD, Anderson DK, Howland DR: **Identification, characterization and function of aggrecan in the adult spinal cord.** *Soc for Neurosci Abst #29519* 1999, **25**(1):750.
49. Titsworth WL, Onifer SM, Liu NK, Xu XM: **Focal phospholipases A2 group III injections induce cervical white matter injury and functional deficits with delayed recovery concomitant with Schwann cell remyelination**. *Exp Neurol* 2007, **207**(1):150-162.
50. Onifer SM, Grover VP, Vargas C, White LA, Gomez AM, Bunge MB, RP: B: **Schwann cell, carbon filament, and collagen grafts placed in the adult rat cervical spinal cord to promote chronically injured axon regeneration.** *Soc Neurosci Abstr* 1997, **23**:1727.
51. Kwon BK, Liu J, Oschipok L, Teh J, Liu ZW, Tetzlaff W: **Rubrospinal neurons fail to respond to brain-derived neurotrophic factor applied to the spinal cord injury site 2 months after cervical axotomy**. *Exp Neurol* 2004, **189**(1):45-57.
52. Miwa H, Gerken T, Huynh T, Flory D, Hering T: **Mammalian expression of full-length bovine aggrecan and link protein: formation of recombinant proteoglycan aggregates and analysis of proteolytic cleavage by ADAMTS-4 and MMP-13.** *Biochim Biophys Acta* 2006, **1760**(3):472-486.
53. Anderson KD: **Targeting recovery: priorities of the spinal cord-injured population**. *J Neurotrauma* 2004, **21**(10):1371-1383.

Appendices.

1. Snow, D. M., updated biosketch

BIOGRAPHICAL SKETCH

Provide the following information for the key personnel and other significant contributors in the order listed on Form Page 2.

Follow this format for each person. DO NOT EXCEED FOUR PAGES

NAME	POSITION TITLE
Diane M. Snow, PhD	Professor with Tenure
eRA COMMONS USER NAME Diane.Snow	

EDUCATION/TRAINING *(Begin with baccalaureate or other initial professional education, such as*

INSTITUTION AND LOCATION	DEGREE *(if applicable)*	YEAR(s)	FIELD OF STUDY
The University of Akron, Akron, OH	B.S.	1982	Biology/German
The University of Akron and NEOUCOM	M.S.	1985	Neuroscience
Case Western Reserve University, Cleveland,	Ph.D.	1989	Neuroscience
Case Western Reserve University, Cleveland,	PostDoc	1990	Neuroscience

A. Personal Statement

This CV serves as an update for a Progress Report to the DOD;
"A Better Way to Excise Inhibitory Molecules (CSPGs) from a Spinal Cord Injury Scar to Promote Regeneration" SC090248

B. Positions and Honors

1984-1986	Technician, Cleveland Clinic Foundation, Dept. of Brain and Vascular Research
1986-1990	Brumagin Fellowship for Spinal Cord Injury Research
1991-1994	NIH National Research Service Award, F32
1992-1993	Macalester College, Minneapolis, MN, Faculty
1994-1996	Research Assistant Professor, The University of Minnesota, Dept. of Cell Biology and Neuroanatomy, Minneapolis, MN
1996-2002	**Assistant Professor**, Dept. of Anatomy and Neurobiology, The Univ. of Kentucky, Lexington, KY
2002-2008	**Associate Professor with Tenure**, Dept. of Anatomy and Neurobiology, The Univ. of Kentucky, Lexington
2003-04	Wethington Research Award
2001-2007	**Faculty Associate, Spinal Cord and Brain Injury Research Center**, Univ. of KY, Lexington, KY
2007	National ACE Network Leadership Award (Advancement of Women in Higher Education) for "an outstanding, innovative, and visionary leadership program – *Circles of Power*"
2008	Kentucky Academy of Sciences - *Superlative Award, Distinguished University Scientist*
2008-pres	**Professor and Endowed Chair**, Spinal Cord and Brain Injury Research Center (SCoBIRC); Dept of Anat and Neurobiology, The University of Kentucky, Lexington, KY
2007-11	Wethington Research Award
2008; 09	Abraham Flexner Master Educator Award *(2011 pending)*

2009	Society for the Promotion of Undergraduate Research (SPUR) - *Excellent Undergraduate Research Mentor Award*
2010	Sarah Bennett Holmes Award, contribution to development of women professionals, *Women's Forum*
2011-12	Southeast Conference Academic Consortium – Administrative Leadership Development Program Fellow
2011-pres	Board of Directors and Advisory Board member, Exomedicine Institute, NASA-funded zero-gravity medical research
2012-pres	Advisory Board, *Living Arts and Science Center,* Lexington, KY
2013-pres	Member, Omicron Delta Kappa leadership society
2014	Co-Chair/Organizer – National Conference on Undergraduate Research

C. Peer-Reviewed Publications (since 2002)

24. **Snow, D. M.**, Smith, J.D., and. Gurwell, J. A. (2002), Binding characteristics of chondroitin sulfate proteoglycans and laminin-1, and correlative neurite outgrowth behaviors in a standard tissue culture choice assay. *J. Neurobiol* 51:285-301.
25. Hynds D. L. and **Snow D. M**. (2002) A semi-automated image analysis method to quantify neurite preference/axon guidance on a patterned substratum. *J. Neurosci. Methods* 121(1):53-64.
26. Hynds, D. L., Inokuchi, J-i, and **Snow, D.M**. (2002) L- and D- threo-1-phenyl-2-decanoylamino-3-morpholino-1-propanol (PDMP) inhibit neurite outgrowth from SH-SY5Y cells. *Neuroscience* 114(3):731-744.
27. Johnson, W.E., Caterson,B, Eisenstein, S.M., Hynds, D.L., Roberts, S. and **Snow, D. M.** (2002) Human intervertebral disc aggrecan inhibits nerve growth in vitro. *Arthritis and Rheumatism* 46(10):2658-2664.
28. **Snow, D.M**., Smith, J.D., Cunningham, A. T., McFarlin, J., and Goshorn E.C. Neurite elongation on chondroitin sulfate proteoglycans is characterized by axonal fasciculation. (2003) *Exp. Neurol*. 182:310-321.
29. Hynds, D.L., Spencer, M., Andres, D., and **Snow, D.M**. (2003) Rit promotes MEK-independent neurite branching in human neuroblastoma cells. – *J. Cell Sci*. 116:1925-1935.
30. Carman, H.M., Booze, R.M., **Snow, D.M**., and Mactutus, C.F. (2003) Proximal versus distal cue utilization in preweanling spatial localization: The influence of cue number and location. *Phys.& Behav*., 79(2):157-165.
31. Hynds, D.L., Rangappa, N., Beest, J.T., **Snow, D.M**., Rabchevsky, A.G. (2004) Microglia enhance dorsal root ganglion outgrowth in Schwann cell cultures. *Glia* 46:218-226.
32. Foltz, T., Carman, H.M., Welch, M.A., **Snow, D.M**., Strupp, B.J, Booze, R.M and Mactutus, C.M. (2004) Prenatal IV. Cocaine and the HR-OR: a dose-response study. *Int'l J. Develop. Neurosci. 22:285-296.*
33. **Snow, D.M**., Smith, J.D., Booze, R.M., Welch, M., and Mactutus, C.F. (2004) Cocaine-induced inhibition of outgrowth in locus coeruleus neurons is related to gestational exposure period and offspring sex. *Int'l J. Develop. Neurosci.* 22:297-308.
34. Dey, S.**,** Booze, R.M., Mactutus, C.F. and **Snow, D.M**. (2006) Specificity of prenatal cocaine on inhibition of locus coeruleus neurite outgrowth. *Neurosci.* 139(3):899-907.
35. Booze, R.M., Wallace, D.R., Silvers, J.M., Strupp, B.J., **Snow, D.M**., and Mactutus, C.F. (2006) Prenatal cocaine exposure alters alpha2 receptor expression in adolescent rats. *BMC Neuroscience* 2006, 7**:**33
36. Heron, P., Sutton, B., Curinga, G.M., Smith, G. M. and **Snow, D.M**. (2007) Localized gene expression of guidance molecules in a co-culture model to direct axonal growth. *J. Neurosci. Meth*., 159(2):203-214.

37. Dey, S., Mactutus, C.F., Booze, R.M., and Snow, D.M. (2007) Prenatal cocaine exposure induces apoptosis in locus coeruleus neurons *in vitro* by altering the bax/bcl-2 ratio and through caspase-3 apoptotic signaling. *Neurosci.* 144(2):509-521.
38. Curinga, G.M., **Snow, D.M.**, Mashburn, C., Kohler, K., Thobaben, R., Caggiano, A.O., Smith, G.M. (2007) Mammalian-produced chondroitinase AC mitigates axon inhibition by chondroitin sulfate proteoglycans. *J Neurochem*. 102(1):275-288.39.
39. Dey, S. and **Snow, D. M**. (2007) Prenatal cocaine exposure induces apoptosis in locus coeruleus neurons through TNF-a mediated induction of Bax and JNK-1-MAPK pathways. *J Neurochem*. 103(2):542-556.
40. Dey, S. and **Snow, D. M**. (2007) Prenatal cocaine exposure induces apoptosis in locus coeruleus neurons through TNF-a mediated induction of Bax and JNK-1-MAPK pathways. *J Neurochem*. 103(2):542-556.
41. Zhang, G. ,R.-L. Lin, M. Wiggers, D.M. Snow, and L.-Y. Lee (2008) Altered expression of TRPV1 and sensitivity to capsaicin in pulmonary myelinated afferents following chronic airway inflammation in the rat. J. Physiol. (Lond) 586:5771-86. PMID: 18832423.
42. Curinga G, **Snow DM,** Smith GM. (2008) Mechanisms regulating interpretation of guidance cues during development, maturation, and following injury. [Invited Review] *Reviews in the Neurosciences* 19 (4-5): 213-26. PMID: 19145984.
43. **Snow, DM** (2011) "Pioneering studies on the mechanisms of axonal regeneration. Dev Neurobiol. 71(9):785-9. PMID: 21938765.
44. Lin S, Liu M, Son YJ, Timothy Himes B, **Snow DM**, Yu W, Baas PW. (2011). Inhibition of Kinesin-5, a microtubule-based motor protein, as a strategy for enhancing regeneration of adult axons. *Traffic* 12(3):269-86. doi: 10.1111/j.1600-0854.2010.01152.x. Epub 2011 Jan 14.
45. Beller, J.A., Kulengowski, B., Kobraei, E., Curinga, G.M., Calulot, CM, Bahrami, A., Hering, TM and **Snow, DM**. Comparison of sensory neuron growth cone and filipodial responses to structurally diverse aggrecan variants, *in vitro*. *Exp. Neurol. (2013*) http://dx.doi.org/10.1016/j.expneurol.2013.02.012.
46**. Snow, DM**., 'Designer molecules', International Innovation, Issue 123: Health partnership, January 2014, Research Media, UK, pp 88-90; ISSN 2051-8552.
47. **Snow DM**. Commentary on: "Facilitating transparency in spinal cord injury studies using data standards and ontologies". Neural Regen Res. 2014;9(1):8-9.
48. Beller JA, Hering TM, **Snow DM**. (2014) "Techniques For Examining The Effect Of Substrate Bound Proteoglycans On Neurite Outgrowth In Vitro." Neuromethods: Extracellular Matrix. Humana Press.
49. Beller JA, Hering TM, **Snow DM**. (2014) "High-throughput Quantitative Assay for Analyzing Neurite Outgrowth on a Uniform Substratum: The Cell-Substratum Assay." Neuromethods: Extracellular Matrix. Humana Press.
50**. Snow, D.M** (2014) Neuronal growth cones and regeneration: gridlock within the extracellular matrix. Neural Regeneration Research 9(4):341-342.
51. Beller, JA, and **Snow, DM**. (2014). Proteoglycans: Road Signs for Neurite Outgrowth. *In: Neuronal Growth Cones and Regeneration: Gridlock within the Extracellular Matrix*. *Ed. D. Snow* – Neural Regeneration Research, February, 9 (4): 358-370

D. Research Support (since 2008)

Proudfoot Family Foundation – May 2014-
Support through proceeds from annual 10K race
Minneapolis, MN

Endowed Chair
Kentucky Spinal Cord and Brain Injury Research Trust
PI, D. Snow (2008-present)

“Recombinant aggrecan variants having specifically modified CS chains and their regulation of axonal regeneration” - ACTIVE
P.I. D. Snow, 1/15/11-1/14/13
Agency: Kentucky Spinal Cord and Head Injury Research Trust
Aims: 1) To use shRNA to knock down specific biosynthetic enzymes in the CS synthesis pathway to produce modified CS chains on recombinant aggrecan (a CSPG). These specifically modified CS chains will be analyzed to confirm the nature of the predicted modification; and, 2) To determine the ability of CSPGs engineered in Aim 1 to inhibit neurite outgrowth.

"A Better Way to Excise Inhibitory Molecules (CSPGs) from a Spinal Cord Injury Scar to Promote Regeneration"
P.I. D. Snow, 9/30/10-2/28/14
Agency: Dept of Defense – DOD-CDMRP
Aims: 1) To determine if aggrecanase and chondroitinase-mediated degradation of CSPGs, either purified, or produced by primary rat cortical astrocytes, will foster neurite outgrowth in vitro; and 2) To determine if aggrecanase and chondroitinase-mediated degradation of CSPGs in a rat spinal cord injury model in vivo, will result in improved axonal regeneration and recovery of function.

“Designer PGs for CNS Injury”
P.I. D. Snow, 9/1/07-2/28/14 (NCE)
Agency: NIH, NINDS; 1R01NS053470-01A2
Aims: Identification and manipulation of inhibitory microdomains of glial scar chondroitin sulfate proteoglycans in vitro focusing on cortical astrocytes interactions with sensory neurons.

"Role of TRPV1 in Airway Hypersensitivity Induced by Inflammation"
PI: Lu-Yuan Lee; Co-I: D. Snow, 9/1/09 - 8/31/11
Agency: NIH - National Heart, Lung and Blood Institute; 1R01 (HL096914-01)
Aims: To investigate the role of TRPV1 in the development of airway hypersensitivity when chronic airway inflammation is induced by allergen sensitization.

“Designer PGs for Spinal Cord Injury”
PI - D. Snow; 11-1-07 to 10-31-010
Agency: Christopher and Dana Reeve Foundation
Aims: identification and manipulation of inhibitory microdomains of glial scar neurocan in vitro and in vivo using shRNA technology.

“PGs, Monastrol, and Regeneration”
P.I.s - D. M. Snow and P. Baas, 12-31-08 to 12-30-09
Agency: Christopher and Dana Reeve Foundation
Aims: To determine if the kinesin-5 inhibitor, monastrol, attenuates CSPG-induced axonal inhibition.